FORUM-ARTICLE

Resisting antimicrobial resistance: Lessons from fungus farming ants.

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ABSTRACT

Attine ants use antimicrobials produced by commensal bacteria to inhibit parasites on their fungal gardens. However, in this agricultural system, antimicrobial use does not lead to overwhelming resistance, as is typical in clinical settings. Mixtures of continually-evolving antimicrobial variants could support this dynamic.

Symbiotic antiparasite defence strategies.

In an antimicrobial-mediated evolutionary arms race, the synthesis and release of antimicrobials evolves in one group of organisms and their competitor organisms counter this

by evolving antimicrobial resistance. Within such a milieu of intense selection and counter-selection, not only will participating organisms evolve, but so too will the antimicrobials themselves.

Fungus-growing ants (tribe: *Attini*) have cultivated specific fungi as food for 60 million years [1]. Their co-evolutionary interdependence is so refined that, for many attine species, their fungal cultivars are not found outside this symbiotic association [1, 2]. The fungi need specific microclimates and nutrition provided by the ants and, in turn, constitute the ants' sole food source. Other fungi, of the genus *Escovopsis*, can invade the cultivated fungus and, because the ants rely entirely on cultivated fungus for food, this parasitism is detrimental to the ants [2]. To counteract these parasitic fungi, ants have evolved multiple strategies. One is a tripartite mutualistic relationship, within which the ants host antimicrobial-producing bacteria on their bodies to protect their fungal cultivar. Many of these bacteria have coevolved with their hosts, producing antimicrobials to inhibit the parasitic fungi whilst in return the ants provide them with nutrition and a microclimate suitable for growth [3]. The parasitic fungi compete with the ant-associated bacteria, as both depend upon the same fundamental source from which they directly, or indirectly, derive nutrition (BOX 1).

Thus, a question arises: Why are these antimicrobials still effective in controlling the parasitic fungi even after millions of years, whilst pharmaceutical antimicrobials are rendered ineffective within a few decades?

Antimicrobial heterogeneity through the evolution of gene clusters.

To control the parasitic *Escovopsis*, the ants house antimicrobial-producing strains of *Pseudonocardia* and *Streptomyces* bacteria on their cuticle. *Streptomyces* strains inhabiting attine clades are acquired via environmental sampling and are, thus, not associated with

specific taxa, but the *Pseudonocardia* are non-randomly associated with attine species and display co-speciation at higher taxonomic levels [4]. Ant-associated *Streptomyces* produce the antimicrobials candicidin and antimycin [5 and references within]. Variants of antimycin produced by *Streptomyces* differ in ultraviolet absorbance profile, liquid chromatography retention time and mass-to-charge (m/z) ratio [5], and are produced by 14 gene clusters (ranging from 15 to 17 genes). The clusters share a region which synthesises the dilactone core common to all antimycin analogues [5, 6]. Therefore, such gene clusters (originating from one ancestral cluster) would support the synthesis of different antimicrobial variants [5, 6]. A similar organisation has been identified in the *Pseudonocardia* spp., which produce multiple, structurally-similar antimicrobials such as dentigerumycin, gerumycin A, gerumycin B and gerumycin C, as well as a polyene antifungal, nystatin P1 [4, 7]. The structural similarity of dentigerumycin, gerumycin A, gerumycin B and gerumycin C, along with region-specific similarity of two of the three clusters responsible for their synthesis, suggests they derive from a single ancestral cluster that diversified in response to selection from antimicrobial-resistant organisms (Figure 1). Several regions of the gene clusters producing these antimicrobials are flanked by mobile genetic elements (transposases, integrases, endonucleases), indicating horizontal gene transfers play a role in their recombination [7]. Such mobile genetic elements could also generate variation within the gene cluster of one clonal line via transference in and out of the cluster. Similarly, in the genomes of the *Pseudonocardia* phylotypes Ps1 and Ps2, novel nystatin P1-like compounds are encoded by at least 14 biosynthetic gene clusters sharing multiple common genes [8]. Thus, it seems likely that variability in effectiveness of variants of antimycin produced by *Streptomyces* and variants of nystatin and dentigerumycin produced by *Pseudonocardia* is a result of constant variation in the gene clusters producing them.

Escovopsis has evolved countermeasures; *in vitro* tests suggesting it can develop resistance to antimicrobials produced by *Pseudonocardia*, even if the majority of wild populations remain susceptible to them [9]. *Escovopsis* is an obligate parasite of the fungus cultivar. This habitat specificity exerts further selection pressure on the parasite to develop resistance to antimicrobials produced by the ant-associated bacteria. Furthermore, recent discovery of two specialised secondary metabolites produced by *Escovopsis* has offered new insight regarding antimicrobial-mediated antagonism between parasite and mutualist triumvirate [2]. Both metabolites inhibit *Pseudonocardia* growth and one, shearinine D, degrades ants' *Escovopsis* weeding efficiency and, at high concentrations, is lethal to them. It may well be that a similar pattern of gene cluster evolution might be present in the *Escovopsis* as described in *Pseudonocardia* and *Streptomyces.*

Comparing arthropod and human strategies of antimicrobial usage.

If modification of antimicrobial-synthesising gene clusters is amplified in the presence of the parasite, the diversity of bacterially-synthesised antimicrobials suggests that there is continuous selection pressure on the bacteria to evolve new variants. They have coevolved with ants that provide their nutrition and microclimate, so the ants' continuing existence is necessary for their survival. In this co-evolutionary arms race, novel bacterial antimicrobial compounds can be formed via novel gene cluster rearrangement or mutations. Novel compounds so generated achieve greater or lesser evolutionary success based upon the *Escovopsis* strain antimicrobial susceptibility. This mechanism is best explicated by Red Queen Dynamics [10], by which in the long term, continual evolution of novel antimicrobial compounds would be encouraged thus preventing sympatric populations of *Escovopsis* from acquiring effective antimicrobial resistance.

In the last decades several models and experimental studies based upon them have been developed, lending some support to this hypothesis. Mathematical and clinical trials show that mixing antibiotics results in resistance reduction [11]. Equally, other symbioses of microorganisms with marine invertebrates, insects and plants, have been shown to rely upon antibiotic mixtures diversified by interspecies and intraspecies interactions, and constructed in conjunction with the evolution of biosynthetic gene clusters [12]. The short generation time of bacteria, rapid recombination of clusters plus horizontal gene transfers [9] are further amplified by marked potency variations offered by only slight antimicrobial structural differences [12]. Such mixtures of antibiotics and their derivatives can even reverse antibiotic resistance via molecular (molecular synergy, antagonism, and suppression) and evolutionary interactions (cross-resistance and collateral sensitivity) [11]. Thus, whereas cross-resistance to whole classes of antimicrobial compounds is a feature of clinical antimicrobial applications, intriguingly, there is little evidence of similar effects limiting efficacy of the structurally-similar compounds employed by the attines' symbionts. If cross-resistance is genuinely absent from this system, it would be of great clinical relevance [11 and references within].

When comparing the attine model of natural selection and diversification of antimicrobials to antimicrobial usage in clinical settings, the contrast is striking. Humans use diverse antimicrobials, but they are structurally discrete compounds rather than the diverse range of subtle variants utilised by the ants and their mutualists. The humans' strategy is also different; employment of discrete antimicrobials as means of rapid pathogen elimination rather than one facet of a long-term strategy of progressive inhibition. Finally, and brutally, the attines have no ethical constraints: not all individuals involved in this arms race must survive.

Bearing this in mind, although all anthropogenic antimicrobials have natural blueprints, it could be that use of structurally-discrete antimicrobials has outlived its usefulness. A new strategy of *in vitro* antimicrobial-mediated arms race simulations would permit evaluation of gene cluster response and emulate an evolutionary approach to antimicrobial generation and utilisation which has served the attines and their allies for 60 million years.

BOX 1

Attine ants, allies, enemies and coevolved crypts. More derived clades of attine ants such as the leaf-cutting ants (*Atta* spp. and *Acromyrmex* spp.) specialise in the cultivation of *Leucoagaricus* spp. fungi. In this mutualistic association *Leucoagaricus gongylophorus* is an obligate cultivar, and forms the ant colony's dominant food source [1]. The fungus is vertically transmitted from colony to colony by the gynes (female reproductive ants) when they first establish their own colonies. The *Leucoagaricus* cultivar may be parasitised by fungi of the genus *Escovopsis* [2]. In response, the ants house multiple antimicrobial-producing bacteria on their cuticle in coevolved cuticular crypts and specialised exocrine glands. The two Actinobacteria predominantly associated with the ants are *Pseudonocardia* and *Streptomyces* [4]. Bacteria are also carried by the gynes on their mating flights and transmitted to offspring colonies [3]. A phylogenetic rooted-tree reconstruction of all known fungus-growing ants showed that the crypts are specifically evolved to house the Actinobacteria. They are morphologically different and differently located on the body of ancient paleo-attines, more basal attine genera and in the later evolved leaf-cutting attines [3].

CAPTION

Figure 1. Summary of the interactions among symbionts of *Acromyrmex* sp. leaf-cutting ants and microorganisms living in their nest. Ants grow the mutualist (green arrows) fungi *Leucoagaricus* spp on cut leaf fragments to provide their sole food source. The parasitic fungus *Escovopsis* also feeds off *Leucoagaricus* (antagonism, red lines). Mutualistic bacteria, *Pseudonocardia* (Phylum Actinobacteria) live on the ants, fed via subcuticular glands and, in return, provide antimicrobial compounds to kill the parasite *Escovopsis* (antagonism). *Escovopsis* counteracts the defensive mutualists by either evolving resistance to the antimicrobials produced by the *Pseudonocardia* or via producing antimicrobials such as melinacidin and shearinine that inhibit *Pseudonocardia*. The evolution of resistance in *Escovopsis* to antimicrobials produced by *Pseudonocardia* induces selection pressures on the bacterial gene clusters (dotted pale blue arrow), causing the evolution of antimicrobial gene clusters harboured in *Pseudonocardia*, via rearrangement of the gene clusters or via positively selected mutations in the gene clusters, thus inducing synthesis of novel antimicrobial compounds.

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